# Using the Acetyl Bromide Assay To Determine Lignin Concentrations in Herbaceous Plants: Some Cautionary Notes

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The acetyl bromide assay was developed to provide a rapid and sensitive method for quantifying lignin in woody plant species. The original procedure cautioned against prolonged reaction times and advised keeping the reaction temperature at 70 °C to prevent excessive carbohydrate degradation that would skew the absorption spectra. Characterization of the reaction conditions revealed that the acetyl bromide reagent readily degrades xylans, a prominent polysaccharide group within all lignified plants. This degradation results in increased absorbance in the 270−280 nm region that is used to quantify lignin. The degradation of xylans is temperature dependent and is exacerbated by the addition of perchloric acid. Lowering the reaction temperature to 50 °C and increasing the reaction time from 2 to 4 h allows complete lignin solubilization but minimizes degradation of the xylans.

**Keywords:** *Acetyl bromide lignin assay; lignin; cell wall; xylans; alfalfa; corn*

## INTRODUCTION

The acetyl bromide based lignin assay was originally developed to provide a rapid yet sensitive method of determining lignin concentrations in small wood samples. Johnson et al. (1961) developed a spectrophotometric method that was easy to perform yet sensitive enough to determine lignin concentrations in relatively small sample sizes (3−6 mg) of woody plant material. It has since been modified a number of times mostly to adapt the procedure to herbaceous plants (Morrison, 1972a,b; Fukushima et al., 1991). Recent modifications have utilized perchloric acid to aid in the total solublization of plant cell walls, including the cellulose fraction (Iiyama and Wallis, 1988, 1990). Only a small amount of insoluble protein residue remained insoluble. It has also been suggested that wall material from grasses be pretreated with either pyrrolidine/pyridine (80 °C for 18 h) or 0.5 M sodium methoxide (80 °C for 18 h) (Morrison and Stewart, 1995) to remove hydroxycinnamic acids before the lignin is dissolved in the acetyl bromide reagent. This was to remove any potential contribution of wall-bound phenolic acids to the total lignin determination because the spectral characteristics of wall-bound hydroxycinnamic acids have regions that overlap with lignin spectra. This may not be crucial, as approximately half of the total ferulates and virtually all of the *p*-coumaric acid are bound to lignin and should be considered as part of the total macromolecule (Ralph et al., 1994, 1998). Essentially none of the ferulates bound to lignin can be released by the treatments suggested by Morrison and Stewart (1995).

We were particularly interested in using this procedure to determine the lignin content of walls in small amounts of isolated cells and on soluble lignin−carbohydrate complexes that do not lend themselves readily to other types of lignin determinations. However, during these analyses using the procedures of Johnson et al. (1961) as modified by Morrison (1972a,b) and Iiyama and Wallis (1988), we were troubled by inconsistent results and/or lignin values that seemed too high for the given sample. This prompted us to revisit the original method and evaluate some of the key modifications that have been proposed over the years since the method was first introduced. We report here on our findings concerning potential degradation of wall carbohydrates and limitations when using perchloric acid to aid in total wall solubilization.

## MATERIALS AND METHODS

The general procedures used in these experiments followed the protocol of Johnson et al. (1961) as modified by Morrison (1972b) and Iiyama and Wallis (1988). For all experiments, the acetyl bromide reagent was 25% (v/v) acetyl bromide in glacial acetic acid. Plant samples used in these experiments were cell walls isolated from lower internodes of alfalfa (*Medicago sativa* L.) stems at the bud stage and corn (*Zea mays* L.) stem rind material (plants at early silk stage). Cell walls (CW) were isolated from stem samples using a modification of the Uppsala method for cell wall analysis (Theander and Westerlund, 1986; Theander, 1991). Briefly, stem samples were ground (UDY mill, 1 mm screen), sonicated in 80% ethanol (40 mL $g^{-1}$, 10 min), and centrifuged (3000*g*, 15 min), and the supernatant was removed. The ethanol extraction step was repeated for a total of four cycles) followed by a single sonication/wash cycle of chloroform/methanol (2:1; 40 mL $g^{-1}$) and a final rinse of acetone (40 mL $g^{-1}$). Air-dried CW material was suspended in 10 mM potassium phosphate buffer (pH 6.0; 0.02% $NaN_3$; 30 mL $g^{-1}$), heated in a water bath (90−95 °C) for 2 h to gelatinize starch, and, after cooling to 55−60 °C, treated with α-amylase and α-amyloglucosidase as described by Hatfield and Weimer (1995). Samples were maintained at 55−60 °C for both the α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1; Sigma A-3403) and amyloglucosidase (1,4-

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α-D-glucan glucanohydrolase, EC 3.2.1.3; Fluka; 10 IU $g^{-1}$) treatments to inhibit structural polysaccharide hydrolytic activity. Destarched CW residues were washed and recovered as described by Hatfield and Weimer (1995). All wall samples were dried overnight at 55 °C before weighing (20−25 mg) into glass culture tubes (16 × 150 mm) fitted with Teflon-lined screw caps. Carbohydrate samples (20−25 mg) were weighed directly into culture tubes. Culture tubes containing weighed samples were placed in a vacuum desiccator over $P_2O_5$ with the caps off for at least 18 h before analysis. Carbohydrate samples included cellulose (Sigmacell), polygalacturonic acid, 4-*O*-methyl-D-glucurono-D-xylan obtained from Sigma, and a xylan isolated from tobacco [courtesy of Paul Weimer isolated according to the procedure of Eda et al. (1976)]. Dehydrogenation polymers [DHPs, courtesy of J. Ralph, synthesized according to the method of Kirk and Brunow (1988)] were formed from coniferyl alcohol, hydrogen peroxide, and horseradish peroxidase.

The standard analysis procedure for acetyl bromide lignin determination was the same for all samples. Individual samples were removed from the desiccator, 2.5 mL of freshly prepared acetyl bromide reagent was added, and the tubes were capped immediately and heated in dry blocks set at 50, 60, or 70 °C. Some samples also contained 100 $\mu$L of perchloric acid to aid in the total dissolution of the wall material (Iiyama and Wallis, 1988, 1990). Heating times ranged from 15 min to 4 h depending upon the nature of the experiment. After heating, the samples were quantitatively transferred, with the aid of acetic acid, to 50 mL volumetric flasks that contained 10 mL of 2 M NaOH and 12 mL of acetic acid. Hydroxylamine (1.75 mL, 0.5 M) was added to each flask, and samples were diluted to 50 mL with acetic acid. An absorption spectrum (250−350 nm) was obtained for each sample and used to determine the absorption at 280 nm. All experiments were run in triplicate with duplicates for each treatment.

CW samples of corn and alfalfa were treated with acetyl bromide reagent for 1, 2, and 4 h and analyzed using the derivatization followed by reductive cleavage (DFRC) procedure of Lu and Ralph (1997a,b) to determine the types and amounts of lignin monomers released. Briefly, after heating with acetyl bromide, the reagent (2.5 mL) was removed by reduced-pressure evaporation. Sample residues were dissolved in 2.5 mL of dioxane/acetic acid/$H_2O$ (5:4:1) and stirred with 50 mg of Zn dust for 0.5 h before being filtered through glass wool. Dichloromethane ($CH_2Cl_2$, 10 mL) was added to the filtrate along with 10 mL of saturated $NH_4Cl$ solution. After through mixing and phase separation, the organic phase was removed and the aqueous phase extracted twice more with $CH_2Cl_2$. All $CH_2Cl_2$ extracts were combined and dried under a stream of argon. Residues were dissolved in 1.5 mL of $CH_2Cl_2$ and acetylated with 0.2 mL of acetic anhydride and 0.2 mL of pyridine. After 90 min, ethyl acetate (2.5 mL) was added and the mixture was evaporated. Several additions of ethanol were used with coevaporation to remove the residual pyridine. Pyridine-free samples were taken up in 200 $\mu$L of $CH_2Cl_2$ for GC analysis. Although this procedure requires several steps and only a fraction of the total lignin is released, it was felt that a comparison of released products (types and amounts) with reaction time would give a good indication of optimum reaction conditions for acetyl bromide lignin determinations.

## RESULTS AND DISCUSSION

Several procedural parameters were initially tested to determine their impact upon the lignin values obtained from standard CW samples (corn rind and alfalfa stem cell walls). These experimental parameters included concentration of acetyl bromide, heating time, heating temperature, perchloric acid addition, and use of hydroxylamine. Acetyl bromide concentration had little impact when the concentration was maintained between 15 and 30% (v/v). It was also determined that addition of hydroxylamine, as described in the original method (Johnson, 1961), gave the most consistent results (due to removal of a polybromide anion that forms during the reaction) and was included as a part of the standard procedure. Experimental parameters of reaction temperature, heating time, and addition of perchloric acid were investigated in greater detail.

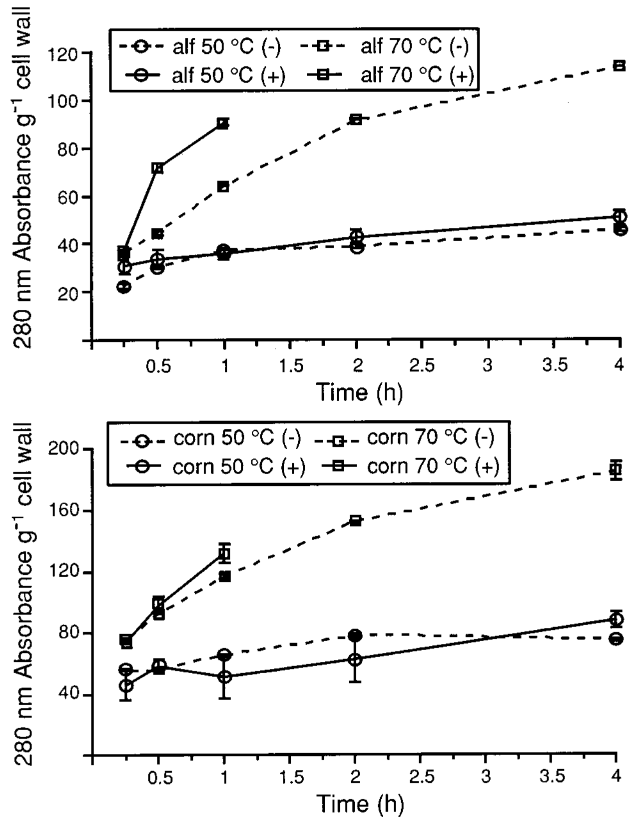


**Figure 1.** Time course of acetyl bromide reaction with corn and alfalfa (alf) walls at different temperatures, with (+) or without (−) perchloric acid.

The impacts of reaction temperature, reaction times, and addition of perchloric acid are summarized in Figure 1. Samples were heated at 50 °C for up to 4 h, at 60 °C for up to 2 h, or at 70 °C for up to 1 h with or without the addition of perchloric acid. Data for the 60 °C trials are not shown, but results were intermediate between the 50 and 70 °C reactions. The inclusion of perchloric acid resulted in higher absorption values as a function of increasing temperature (Figure 1, solid lines). This could be interpreted to mean that higher temperatures or the addition of perchloric acid is required to solubilize all of the wall-bound lignin. Alternatively, the increase in absorbance could be due to increased polysaccharide degradation. Addition of perchloric acid at 50 °C did not cause a large change in the total absorbance as a function of time even out to 4 h (Figure 1). This suggests that factors other than additional solubilization of lignin were responsible for the increase in absorbance.

DHPs formed from coniferyl alcohol, hydrogen peroxide, and horseradish peroxidase were readily soluble in the acetyl bromide reagent, even with limited heating. As might be expected, the perchloric acid and temperature had little impact on total absorbance due to ready solubility in acetyl bromide reagent and the purity of the lignin (Figure 2). All temperatures gave nearly the same absorbance values with only a slight increase when perchloric acid was added to the reaction at 70 °C, suggesting possible oxidative changes in the

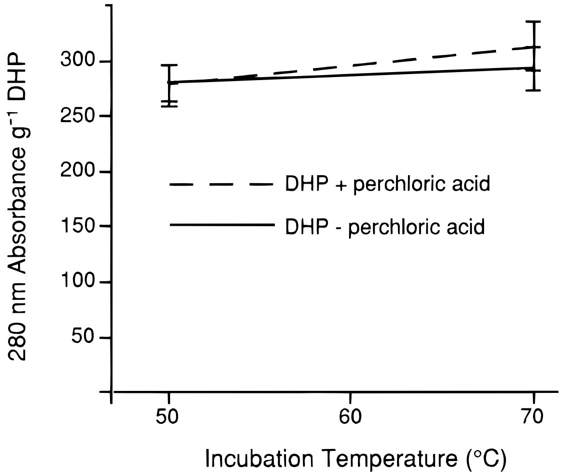


**Figure 2.** Impact of reaction temperatures on the total absorbance of a coniferyl alcohol DHP solubilized in acetyl bromide reagent. Reaction time at 50 °C was 2 h and at 70 °C was 0.5 h.

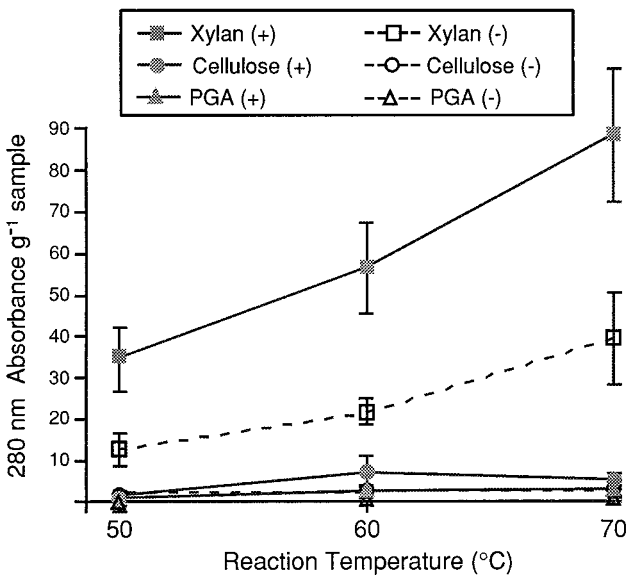


**Figure 3.** Reaction of CW polysaccharides with acetyl bromide reagent and total absorbance generated with increasing reaction temperatures. Solid lines represent samples with perchloric acid added, and dashed lines were without perchloric acid. Heating times were 2 h at 50 °C, 1 h at 60 °C, and 0.5 h at 70 °C.

lignin subunits. These changes were minor compared to the increase in absorbance when a CW sample was evaluated.

To address the question of possible carbohydrate degradation in acetyl bromide reagent, structural polysaccharides were treated with acetyl bromide following the same regime as for CW samples. Xylans were particularly susceptible to degradation in the acetyl bromide reagent (Figure 3). Two different sources of xylan were evaluated to ensure that changes in absorbance were not due to lignin contaminants within the xylan preparations. Increased temperature accelerated this degradation, which was further enhanced by the addition of perchloric acid. Although the absorption spectra for degraded xylans were different from those for lignin, extending further into the higher wavelengths, there was a strong maximum absorbance at 280 nm (Figure 4) that coincides with the absorption maximum used for lignin. It is likely that a good deal of the increased

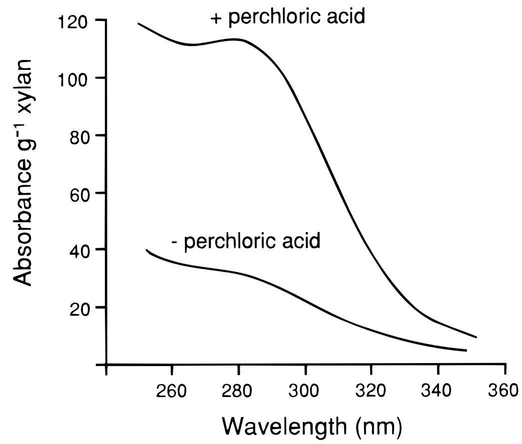


**Figure 4.** Absorbance spectra of xylans treated with acetyl bromide reagent with (+) or without (−) perchloric acid for 0.5 h at 70 °C.

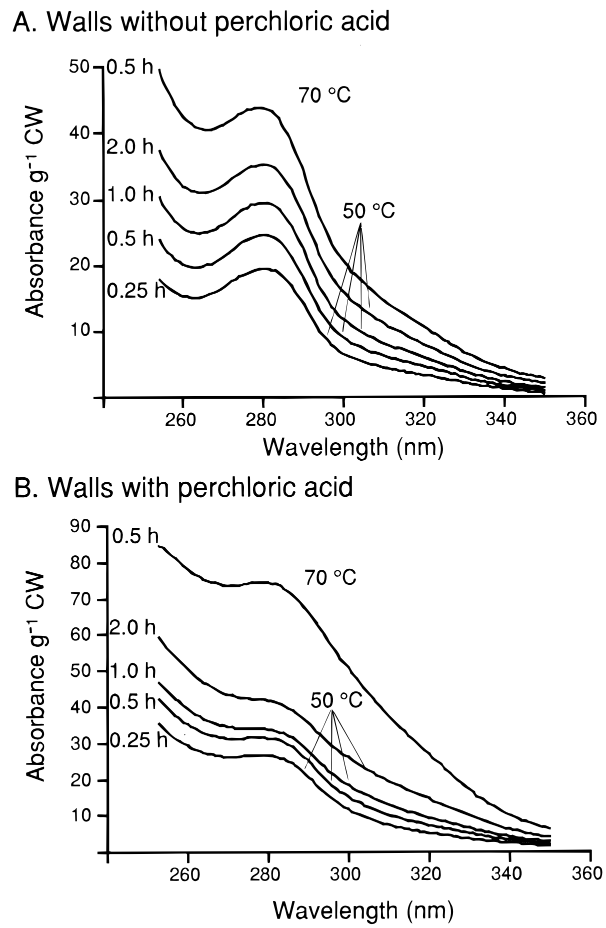


**Figure 5.** Absorbance spectra of alfalfa walls treated with acetyl bromide reagent: (A) spectra generated without the addition of perchloric acid; (B) spectra generated with the addition of perchloric acid.

absorbance observed for wall samples at higher temperatures and especially in the presence of perchloric acid was due to xylan degradation. This can be clearly seen in spectral scans of alfalfa walls treated with acetyl bromide reagent for increasing times and different temperatures (Figure 5). Without perchloric acid, at 50 °C, the spectra show a clean, fairly sharp maximum at 270−280 nm that increases with additional reaction time (Figure 5A). Spectra at 70 °C show the same general characteristics. In contrast, the addition of

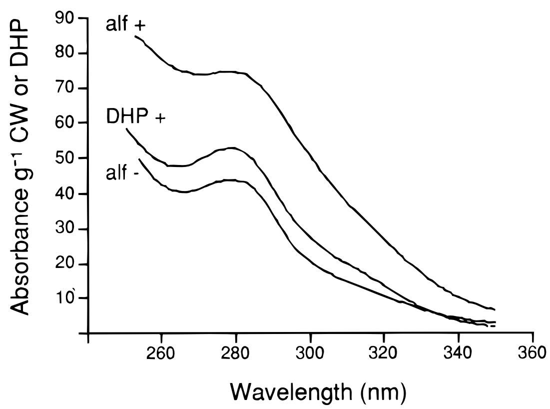


**Figure 6.** Spectral comparison of alfalfa CWs and coniferyl alcohol DHP treated with acetyl bromide reagent at 70 °C with (+) or without (−) perchloric acid. The DHP spectrum was normalized to roughly the same level of lignin as in the cell wall sample.

perchloric acid changes the spectra even at 50 °C with the increased reaction time, resulting in a broadening of the maximum and increased absorbance at higher wavelengths (Figure 5B). At 70 °C, the impact is even more dramatic (Figure 5B). Compared to the spectra obtained from xylan, the changes in the perchloric acid treated samples appear to be the result of xylan degradation and not lignin solubilization. Earlier work by Reeves (1993) had also indicated that carbohydrates may be interfering with the acetyl bromide lignin assay, particularly when perchloric acid was used to aid in wall solubilization. Our results differ in that there was no significant reaction of pectic carbohydrates (polygalacturonans from Sigma, Figure 3, or pectic carbohydrates isolated from alfalfa data not shown) with acetyl bromide reagent.

Attempts were made to identify the degradation products from acetyl bromide treated xylans. NMR analysis indicated that the products were oligomeric/polymeric in nature with characteristics resembling furfurals and furfuryl alcohol. A comparison of xylan before and after acetyl bromide treatment reveals several changes in the spectra. Most noticeable was the increased complexity in the 3−5 ppm region, the detection of compounds with double bonds (6−8 ppm region), and the formation of aldehydes (9−10 ppm). Although it was not possible to identify the compounds responsible for these changes, it is clear that formation of molecules with double bonds and aldehyde functional groups would contribute to absorbance in the 250−300 region. The material did not appear to be monomeric in nature, and GLC analysis of the degradation products did not reveal monomers; trace amounts of furfural and furfuryl alcohol were produced. It would appear that the degradation products are oligosaccharides that have the reducing end modified.

Coniferyl alcohol DHPs treated with acetyl bromide reagent containing perchloric acid produce spectra that do not show the broadening seen with cell wall samples (Figure 6) or with xylans (Figure 4) treated under the same conditions. This would confirm that problems with higher temperatures and perchloric acid are the result of carbohydrate degradation. On the basis of the release of lignin products using the DFRC method of lignin monomer analysis (Lu and Ralph, 1997a), 2−4 h of treatment at 50 °C provides optimal solubilization of lignin yet minimizes the production of polysaccharide degradation products as confirmed by GC/MS of lignin monomers.

## CONCLUSIONS

The acetyl bromide assay for lignin is a convenient procedure for small samples that may not be suitable for lignin methods dependent upon the production of an insoluble residue. However, xylan degradation should be considered as a possible interference that would overestimate total lignin concentrations. By using a lower temperature (50 °C) and increasing the reaction time from 2 to 4 h, depending on the difficulty of wall solubilization, carbohydrate degradation can be minimized. On the basis of the release of lignin monomers using the DFRC method for lignin analysis, 2−4 h treatment at 50 °C appears to provide an optimum solubilization of lignin yet minimizes the production of polysaccharide degradation products. On the basis of the findings of these experiments, it is recommended that perchloric acid be left out of the acetyl bromide reagent and that the reaction temperature be decreased to 50 °C (2−4 h).

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